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Quantification of total phenol, flavonoid content and pharmacognostical evaluation including HPTLC fingerprinting for the standardization of *Piper nigrum* Linn fruits

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ABSTRACT

Objective: To carry out the physicochemical and phytochemical standardization with high performance thin layer chromatography fingerprinting of *Piper nigrum* L. (*P. nigrum*) fruits in order to ascertain the standard pharmacognostical parameters of this king of spices.

Methods: Many standardization parameters like extractive values, total ash value, water soluble ash value and acid insoluble ash, moisture content, loss on drying and pH values of *P. nigrum* L. fruits were analyzed. The method of Harborne was adopted for the preliminary phytochemicals screening. Analysis of total phenolic and flavonoid contents, pesticides residues, aflatoxin and heavy metals were also performed. CAMAG-high performance thin layer chromatography system was used for fingerprinting of methanolic extract of *P. nigrum* L. fruits.

Results: The results of phytochemicals testing indicated the presence of carbohydrates, phenolic compounds, flavonoids, alkaloids, proteins, saponins, lipids, sterols and tannins in various solvent extracts. Total phenolic and flavonoid contents in methanolic extract were found to be 1.728 1 mg/g and 1.087 μg/g, respectively. Heavy metals concentrations were found to be within standard limits. Aflatoxins and pesticides residues were absent.

Conclusions: The outcome of this study might prove beneficial in herbal industries for identification, purification and standardization of *P. nigrum* L. fruits.

1. Introduction

Piper nigrum (*P. nigrum*) (black pepper) is a valuable medicinal plant which belongs to the family Piperaceae. Black pepper is an important and most commonly used spice and regarded as "the king of spices" among various spices[1]. *P. nigrum* is grown in various tropical regions like India, Indonesia and Brazil. *P. nigrum* is commonly known as "Kali Mirch" (Hindi and Urdu), "Pippali" (Sanskrit), "Milagu" (Tamil) and peppercorn, white pepper, green pepper, black pepper, Madagascar pepper (English). Hot and pungent peppercorns are obtained from black pepper. Black pepper is also used as a medicine, a preservative and a flavoring agent in perfumery. Whole peppercorn of *P. nigrum* or its active constituents are used worldwide in different types of foods and sauces and dishes like meat dishes. *P. nigrum* contains major pungent alkaloid known as piperine (1-peperoyl piperidine). *P. nigrum* is used in the treatment of various diseases since many centuries in different

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indigenous systems of medicine as well as folk medicines. It has been used as a common remedy in traditional Unani, Ayurvedic, and Chinese system of medicines[2]. *P. nigrum* and piperine are reported to exhibit wide spectrum of pharmacological activities such as antitumor[3], antidepressant[4], anti-asthmatic[5], antihypertensive and antiplatelets[6], anti-inflammatory[7], antimicrobial[8], antioxidant[9,10], hepatoprotective[11], antidiarrheal[12], immunomodulatory[13], anticonvulsant[14] and analgesic activities[15], *etc*. Many therapeutic activities of this spice are attributed to the presence of piperine apart from other chemical constituents[16].

Piperine has been found to enhance the therapeutic efficacy of many drugs, vaccines and nutrients by increasing oral bioavailability by inhibiting various metabolizing enzymes[17,18]. It is also known to enhance cognitive action and fertility[19], stimulates pancreatic and intestinal enzymes to help in digestion.

In the past, therapeutic potentials of *P. nigrum*, its extracts or its important active chemical constituent ''piperine'' have been published in various international journals. Worldwide researchers have shown a interest in the research on *P. nigrum*. Adulterations, substitution and impurities are the most frequently occurring problems in natural drugs. There are many modern techniques to ensure quality control of medicinal plants and their products. Since, adulteration is a main problem of herbal drugs which can be controlled by using modern scientific sophisticated methods.

Based on these facts and in order to establish the quality control parameters of this valuable spice, the standardization of fruits of *P. nigrum* was performed according to standard methods of World Health Organization (WHO) and guidelines of different pharmacopoeia for herbal drugs. Therefore, current research work was designed to carry out the physicochemical and phytochemical analysis of *P. nigrum* fruits supported by high performance thin layer chromatography (HPTLC) fingerprint of its various extracts. The outcome of this research might be utilized for quality control of this king of spice while standard parameters can be used for identification, purification and to distinguish this valuable spice from the adulterants in academic or industrial organizations.

2. Materials and methods

2.1. Collection and authentication of drug

The fruits of *P. nigrum* Linn were procured from a local supplier of Khari bawli market, Delhi and authenticated by a taxonomist of Jamia Hamdard. For future reference, a sample specimen No. (PN/FP-JNU) was submitted to the Faculty of Pharmaceutical Sciences, Jodhpur National University, Rajasthan, India. The *P. nigrum* fruits were thoroughly washed with water to remove the impurities and dried under shade. The extracts were prepared from the dirt free fruits.

2.2. Preparation of extracts

The fruits of *P. nigrum* were dried in an oven at a temperature less than 60 ℃. The sample was powdered and then passed over sieve No. 14. The dried powdered sample fruits of *P. nigrum* (500 g) were placed in a Soxhlet apparatus on water bath for 6 h for extraction with different solvents like petroleum ether, *n*-butanol, chloroform, and methanol.

The prepared extracts were filtered and dried by evaporation using rotary evaporator from Buchi, Rotavapor R-210, Switzerland and the final extracts were kept at low temperature for further investigations.

2.3. Physicochemical standardization

The standardization of the extracts of *P. nigrum* fruits were carried out as per guidelines of WHO and different procedures in pharmacopeia. The standardization studies on different physicochemical parameters including extractive values in different solvents, total ash value, water soluble ash value, acid insoluble ash value, moisture content, loss on drying (LOD) and pH values of 1% and 10% solutions were carried out. Aflatoxins, pesticides residues and heavy metals in various extracts were determined according to the standard methods[20,21].

2.4. Preliminary phytochemicals testing

The preliminary phytochemical investigations of different solvent extracts of *P. nigrum* fruits were performed as per the reported methods, to detect the various classes of phytoconstituents such as carbohydrates, phenolic compounds, flavonoids, alkaloids, proteins, saponins, lipids, steroids and tannins[22]. Total phenols and flavonoids contents in the fruits extracts were also analyzed by UV spectroscopy as per standard procedures[23,24].

2.5. Determination of total phenolic contents by UV spectrophotometer

Folin Ciocalteu method was used to determine the total phenolic contents of methanolic extract by UV spectrophotometer. An external calibration curve of gallic acid as standard phenolic compound was plotted. The total phenolic contents of the methanolic extract of fruits were calculated with the help of a standard calibration curve[23], and are reported as gallic acid equivalent (mg/g of dry mass).

2.6. Determination of total flavonoid contents by colorimetric method

Aluminum chloride colorimetric method of Chang *et al*. was used to analyze the flavonoid content of methanolic extract of *P. nigrum* fruits[24]. Quercetin (standard flavonoid compound) was used to construct a standard calibration curve. The flavonoid contents in the sample of fruits extract is reported as quercetin equivalent (μg/g of dry mass).

2.7. Development of chromatographic HPTLC fingerprint profile of methanolic extract of P. nigrum

CAMAG-HPTLC system of Switzerland with a Linomat 5

sample applicator was used to obtain HPTLC fingerprinting. HPTLC fingerprint profile of the methanolic extract of *P. nigrum* was developed to confirm the occurrence of different phytopharmaceuticals. Different combinations of solvent systems were tried to obtain an excellent separation and sharp peaks for analysis. The satisfactory resolution for separation of compounds presented in methanolic extract of *P. nigrum* was obtained in the toluene: diethyl ether: dioxane (6:2:1) solvent system.

The investigations were performed in an air-conditioned room maintained at a temperature 22 ℃ and 55% humidity. Precoated silica gel HPTLC aluminium plates 60F-254 (20 cm×20 cm, 0.2 mm thicknesses, 5–6 μm particle size, E-Merck, Germany) were used for chromatographic separation. The extract (5 μL) was spotted as bands of 6 mm width with the help of the auto sampler fitted with a 100 μL Hamilton syringe. The plates were prepared by using solvent system of toluene: diethyl ether: dioxane (6:2:1). The solvent system was transferred to CAMAG Twin Trough plate development chamber lined with filter paper and pre-saturated with mobile phase (30 mL). The resulted plates were air dried and scanned. A spectrodensitometer (Scanner 3, CAMAG) equipped with winCATS planar chromatography manager (version 1.3.0) software was employed for the densitometry measurements, spectra recording and data processing. Absorption/remission was then measured at a scan speed of 20 mm/s. Chromatograms were recorded at the wavelength of 254 and 366 nm. The retention factor (R_f) value of each compound separated on plate and data of peak area of each band were recorded.

2.8. Determination of heavy metal residues

Heavy metals residues *viz*. Cd, Pb, As and Hg in the *P. nigrum* extracts were analysed as per the American Organization of Analytical Chemists (AOAC) official methods of analysis[25].

2.9. Determination of pesticide residues

Gas chromatography-mass spectrometer was used to determine the presence of pesticide residues, including organochlorines, organophosphates and pyrethroids in the extracts by AOAC guidelines[25].

2.10. Aflatoxin analysis

P. nigrum fruits extracts were subjected to aflatoxins analysis by HPLC method as per the AOAC guidelines[26].

3. Results

3.1. Physicochemical standardization

The purity of the drug was checked by determining different physicochemical parameters which included extractive values, total ash value, acid insoluble ash value, water soluble ash value, moisture content, LOD, pH values of 1% and 10% solutions. The results of physicochemical parameters are summarized in Table 1. The moisture content in the fruits of *P. nigrum* was found to be 0.48% which indicated that the drug was properly dried and well stored. LOD of the fruits of *P. nigrum* was found to be 10.23%±1.43%. The pH values of the *P. nigrum* fruits extracts (1% and 10% solutions) were also evaluated with the help of digital pH meter. The pH of the 1% and 10% solutions of drug was found to be 6.23±0.43 and 8.18±0.44 respectively.

Table 1

Results of physicochemical analysis of *P. nigrum* L. (black pepper) fruits.

Physicochemical parameters		Average values (%)
Extractive values	Petroleum ether	10.51±0.62
	Chloroform	8.25±0.56
	n-Butanol	5.34±0.87
	Methanol	12.56±1.32
Ash values	Total ash value	4.31±0.32
	Acid insoluble ash value	0.48±0.44
	Water soluble ash value	3.58±0.54
LOD		10.23±1.43
Moisture contents		0.48±0.44
pH of the drug (1% solution)		6.23±0.43
pH of the drug (10% solution)		8.18±0.44

Values are expressed as mean±SE.

3.2. Preliminary phytochemicals analysis

The results of preliminary qualitative phytochemical screening revealed the presence of alkaloids, carbohydrates, phenolic compounds, flavonoids, proteins, saponins, lipids, tannins and steroids. All these phytochemicals except lipid were found to present in the methanolic extracts. The results of phytochemicals analysis are presented in Table 2.

Table 2

Results of phytochemicals screening of different extracts of the *P. nigrum* L. (black pepper) fruits.

Constituents	Fruits extracts			
	Petroleum ether	Chloroform	*n*-Butanol	Methanol
Alkaloids	Absent	Present	Absent	Present
Carbohydrates	Absent	Absent	Absent	Present
Phenolic compounds	Absent	Present	Absent	Present
Flavonoids	Absent	Present	Present	Present
Proteins and amino-acids	Absent	Absent	Present	Present
Saponins	Absent	Present	Present	Present
Lipids/fats	Present	Absent	Absent	Absent
Tannins	Absent	Absent	Absent	Present
Sterols		Present		Present

3.3. Total phenolic contents

The total phenolic contents of methanolic extract of *P. nigrum* were determined by UV spectrophotometric method. The total content of phenolic compounds was found to be (1.728 1±0.049 0) mg/g of gallic acid equivalent in methanolic extract of *P. nigrum* fruits. The given values are mean±SD of three different determinations.

Table 3

HPTLC fingerprinting profile of methanolic extracts of *P. nigrum* L. (black pepper) fruits.

Wavelength	Solvent systems	No. of peaks	R_f values	Percentage peak area
366 nm	Toluene: diethyl ether: dioxane (6:2:1)	11	0.09, 0.20, 0.29, 0.34, 0.41, 0.44, 0.51, 0.57, 0.71, 0.80, 0.93	15.42, 3.79, 10.68, 6.21, 11.98, 4.90, 7.88, 5.10, 22.83, 8.17, 3.04
254 nm	Toluene: diethyl ether: dioxane (6:2:1)	7	0.51, 0.41, 0.50, 0.58, 0.68, 0.78, 0.83	4.12, 4.54, 19.77, 4.46, 12.43, 45.71, 8.96

3.4. Total flavonoids content

The flavonoid content of methanolic extract of *P. nigrum* wdetermined by UV spectrophotometric method. The total content of flavonoids was found to be (1.087±0.002) µg/g quercetin equivalent in methanolic extract of *P. nigrum* fruits. The given values are expressed as mean±SD of three different determinations.

3.5. HPTLC fingerprint profile of methanolic extract of P. nigrum

HPTLC fingerprinting of methanolic extract of *P. nigrum* fruits was carried out by using toluene: diethyl ether: dioxane (6:2:1) solvent system to confirm the presence of various phytoconstituents in the extract. HPTLC chromatogram showed a total of 11 peaks at different R_f values and peak area at 366 nm (Figures 1 and 2, Table 3) where as 7 peaks were observed in HPTLC chromatogram at 254 nm (Figures 3 and 4, Table 3). The total number of constituents (No. of peaks) in the extract and their R_f is summarized in Table 3 and chromatographic profile had been shown by Figures 1-4.

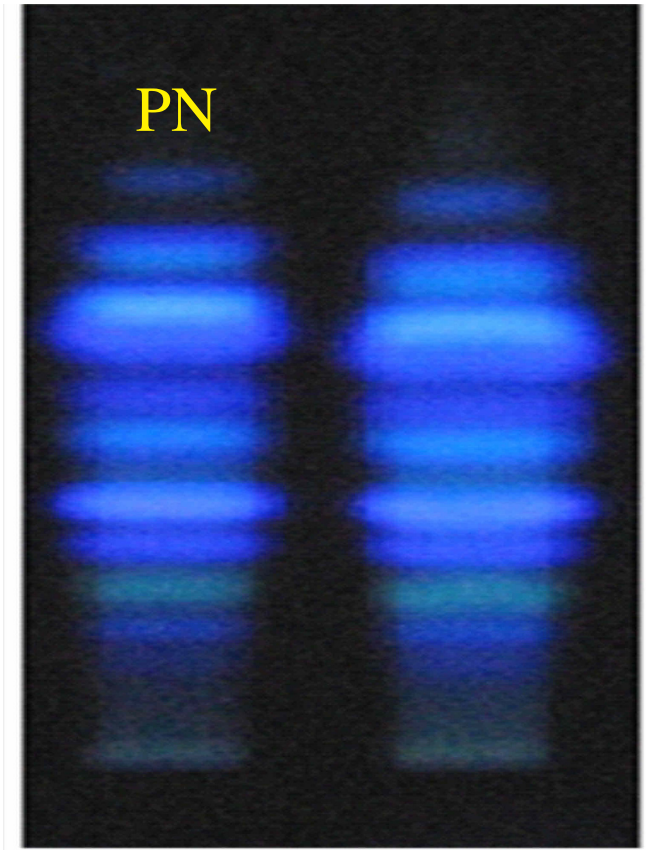

Figure 1. HPTLC photograph of methanolic extract of *P. nigrum* at 366 nm.

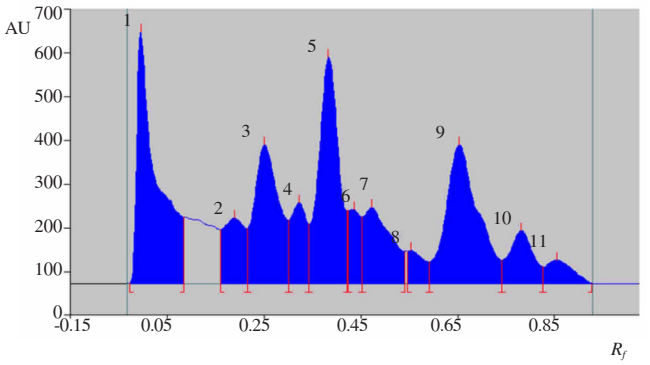

Figure 2. Chromatogram of the methanolic extract of *P. nigrum* at 366 nm.

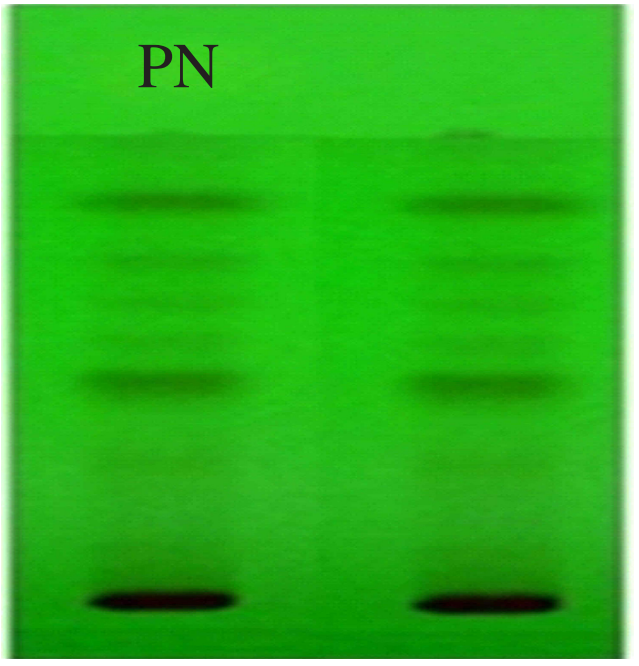

Figure 3. HPTLC photograph of methanolic extract of *P. nigrum* at 254 nm.

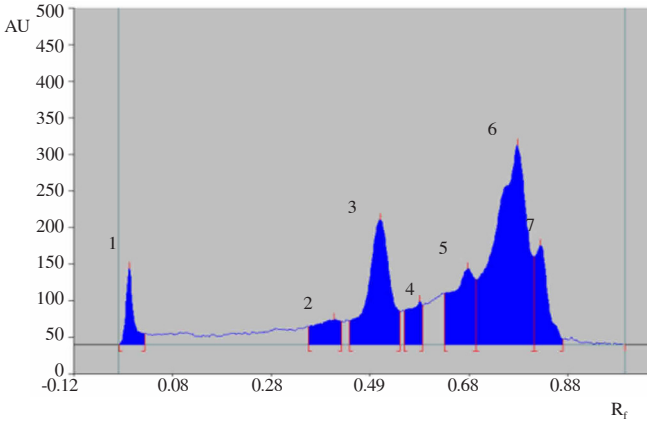

Figure 4. Chromatogram of the methanolic extract of *P. nigrum* at 254 nm.

3.6. Determination of heavy metal residues

The atomic absorption spectrophotometer was used to analyze the heavy metals (Cd, Pb, As, Hg) in the fruit extracts of *P. nigrum*. All essential safety precautions were implemented to avoid potential contamination in the sample according to the AOAC guidelines. The level of Cd concentration was found to be (0.22±0.04) mg/kg. It was lower than the acceptable limit of 0.3 mg/kg as set by WHO guidelines in all the samples. Pb was found to be (0.45±0.05) mg/kg which was much lower than the acceptable limit of 10 mg/kg by WHO guidelines in all the samples. As and Hg were found to be (0.18±0.06) and (0.27±0.06) mg/kg, respectively in the sample of *P. nigrum* fruits extract. Both of these metals were found to be within permissible limits of 0.5 and 1.0 mg/kg, respectively shown in Table 4.

Table 4
Determination of heavy metal residues.

Test parameter	Concentration (mg/kg)
Cd	0.22±0.04
Pb	0.45±0.05
As	0.18±0.06
Hg	0.27±0.06

3.7. Determination of pesticide residues

The presence of 40 pesticide residues like organochlorines, organophosphates and pyrethroids was investigated in the extracts by using gas chromatography-mass spectrometer as per guidelines of AOAC[25]. All 40 pesticides were found to be absent in all the samples of *P. nigrum* fruits extract.

3.8. Aflatoxin analysis

Various aflatoxins *e.g.* B1, B2, G1 and G2 were investigated but were found to be absent in all the samples of *P. nigrum* fruits extract.

4. Discussion

Bioassays can play an important role in the standardization of herbal drugs and their product of therapeutic purpose. Standardization is an essential measurement for ensuring the quality control of the herbal drugs. Different physicochemical parameters are being used to standardize the herbal drugs[27]. The extractive values are used to determine the active constituents. The highest percentage of *P. nigrum* fruits extract was found to be 12.56% in solvent methanol. The analysis revealed that *P. nigrum* fruits have higher concentration of fatty constituents. The ash values were analyzed to check the possible presence of any foreign matters like soil, sand and adherence of water soluble salts to the drug's surface. The ash values of different drugs may vary but this difference lies within narrow limits in the case of the similar drugs. The acid insoluble ash usually contains silica and therefore, high acid insoluble ash is an indicator of the contamination with earthly materials to the drug.

The water-soluble ash indicates the quantity of inorganic elements in the sample. Therefore, the total ash values of the herbal drugs are not always reliable parameters because of the risk of the occurrence of non-physiological materials. Thus, acid insoluble ash was quantified which revealed the lowest content in the extract of the black pepper fruits. Moisture contents and LOD were used to find out the amount of moisture including volatile contents of the tested drug. The hydrolysis of the active ingredients of drug may occur due to higher moisture content in the drug which leads to poor quality and efficacy. The final processes of dryness and removal rate of moisture contents are of great importance. The pH of the extracts showed the acidic and basic compounds concentration. Since, the plants are said to be biosynthetic laboratory for a multitude of chemical compounds. The secondary metabolites are compounds which are responsible for therapeutic efficacy of the drugs. The results of preliminary screening of the phytochemicals in methanolic extract of *P. nigrum* fruits showed the presence of carbohydrates, phenolic compounds, flavonoids, alkaloids, proteins, saponins, lipids, sterols and tannins. Tannins bind to proteins and inhibit to the protein synthesis. Therefore, the current preliminary phytochemicals screening might be proved valuable in the detection and further quantitative analysis of these therapeutically important compounds. The phenolic and flavonoid compounds are important antioxidants which also include antimicrobial, anti-allergic, anti-inflammatory and anticancer agent, *etc*. Phenolic compounds are most widely distributed phytochemicals which are derivatives of pentose phosphate, shikimate, and phenylpropanoid pathways in plants. These secondary metabolites play a vital role in reproduction and growth. These compounds also provide protection against harmful pathogenic microbes and predators[28]. Therefore, quantitative analysis of such vital compounds is extremely significant to determine the quality of drugs. The total quantitative analysis of phenolic and flavonoid contents of *P. nigrum* fruits was carried out by UV spectroscopic method.

The total phenolic and flavonoid contents in the methanolic extract of *P. nigrum* fruits were found to be (1.728 1±0.049 0) mg/g and (1.087±0.002) μg/g respectively. These results showed that the *P. nigrum* fruits extract contain significant quantity of phenolic and flavonoid compounds.

HPLTC has emerged as an important sophisticated analytical tool for the detection, separation, estimation and evaluation of diverse groups of natural products. Chromatographic fingerprint is considered as a rational method for more powerful and effective quality control of herbal drugs and their products[29]. Results of HPTLC fingerprinting of methanolic extract of *P. nigrum* fruits showed the presence of various chemical constituents in the extracts. HPTLC chromatogram showed 11 peaks at different R_f values and peak area at 366 nm in toluene: diethyl ether: dioxane (6:2:1) solvent system whereas 7 peaks were present in HPTLC chromatogram at 254 nm. The number of peaks indicating the number of constituents in the extract. In summary, the fingerprint

images of *P. nigrum* fruits obtained from HPTLC analysis in this study can be referred as standard fingerprints as a reference of authentication, identification, purification, quality control evaluation and to separate the fruits of this king of spices from its adulterants in order to ensure its therapeutic efficacy.

Heavy metals are inorganic materials which are highly toxic to human beings even at very low concentrations. Heavy metals are stored in different parts of plants which enter through the biological cycle of plants. Moreover, the dietary intake of contaminated plants with heavy metals could also lead to dangerous consequences for the health of humans and animals[30]. Various herbal drugs are usually contaminated with the most common toxic metals *viz.* Cd, As, Hg and Pb[31]. Pb is known to cause different types of human diseases and disorders. Kidney damage, anemia, lower sperm count, miscarriage, some neurological disorders and hepatotoxicity at higher concentration of Pb are some important examples of disorders[32]. Cd causes significant toxicity which affects most of organ systems. It was very toxic which impact on the kidney. Acute or chronic Cd exposure causes cancers, anemia, hemorrhagic trauma, respiratory distress, and cardiovascular diseases[33].

Arsenic is a toxic metalloid element which causes many human health problems. Inorganic As is considered more toxic as compared to organic As. Chronic and acute exposures of As result in cancer, neurological disorders, atherosclerosis, hypertension, glycemic index disturbances, renal and liver diseases, reproductive diseases, dermatological disorders and many other health problems[34]. The concentrations of As, Cd, Pb, and Hg analyzed in the extracts of fruits of the *P. nigrum* using atomic absorption spectrophotometer were found to be within permissible limits.

Pesticides are known to cause toxicity in human beings; therefore, all the herbal drugs should be devoid of these substances. A total number of 40 pesticides was tested in the samples but none of the pesticides was in our extracts.

Secondary metabolites produced by fungi are known as mycotoxins[35]. Fungi producing mycotoxins might lead to the contamination of herbal preparations during various stages of processing and production. The most common mycotoxins producing fungi are *Aspergillus parasiticus*, *Aspergillus flavus*, and *Fusarium verticillioides*. *Aspergillus* species produce aflatoxins B1, B2, G1 and G2. These aflatoxins are known to cause liver cancer in human beings[36]. No aflatoxin was detected in the *P. nigrum* fruits extract.

The outcome of these findings might be useful as a diagnostic tool for the standardization of this king of spices. The findings of this study will also be useful for the characterization and establishment of standardization parameters of the fruits of *P. nigrum*. Therefore, the current study will offer adequate knowledge pertaining to therapeutic efficacy of this drug and also in the identification and quality control of this miraculous king of spices.

Conflict of interest statement

We declare that we have no conflict of interest.

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